Selective homocysteine-lowering gene transfer attenuates pressure overload-induced cardiomyopathy via reduced oxidative stress

Ilayaraja Muthuramu, Neha Singh, Ruhul Amin, Elena Nefyodova, Mirjam Debasse, Isa Van Horenbeeck, Frank Jacobs, Bart De Geest

Centre for Molecular and Vascular Biology, Department of Cardiovascular Sciences, Catholic University of Leuven, Leuven, Belgium.

Word count main text body: 3452

Address for correspondence: Bart De Geest MD, PhD
Centre for Molecular and Vascular Biology
Department of Cardiovascular Sciences
Catholic University of Leuven
Campus Gasthuisberg
Herestraat 49 bus 911
3000 Leuven
Belgium
Tel.: 00 32 16 372059
Fax.: 00 32 16 345990
E-mail: bart.degeest@med.kuleuven.be

ABSTRACT

Plasma homocysteine levels predict heart failure incidence in prospective epidemiological studies. We evaluated whether selective homocysteine lowering gene transfer beneficially affects cardiac remodelling and function in a model of pressure overload-induced cardiomyopathy induced by transverse aortic constriction (TAC). Female C57BL/6 *low-density lipoprotein receptor* (*Ldlr*$^{-/-}$) *cystathionine-β-synthase* (*Cbs*$^{+/-}$) mice were fed standard chow (control mice) or a folate-depleted, methionine-enriched diet to induce hyperhomocysteinemia (diet mice). Three weeks after initiation of this diet, mice were intravenously injected with 5 x 10^{10} viral particles of an E1E3E4-deleted hepatocyte-specific adenoviral vector expressing *Cbs* (AdCBS), with the same dose of control vector, or with saline buffer. TAC or sham operation was performed two weeks later. AdCBS gene transfer resulted in 86.4% ($p<0.001$) and 84.6% ($p<0.001$) lower homocysteine levels in diet sham mice and diet TAC mice, respectively. Mortality rate was significantly reduced in diet AdCBS TAC mice compared to diet TAC mice during a follow-up period of 8 weeks (hazard ratio for mortality 0.495, 95% CI 0.249 to 0.985). Left ventricular hypertrophy ($p<0.01$) and interstitial myocardial fibrosis ($p<0.001$) were strikingly lower in control TAC mice and diet AdCBS TAC mice compared to diet TAC mice. Diastolic function in diet AdCBS TAC mice was similar to that of control TAC mice and was significantly improved compared to diet TAC mice. AdCBS gene transfer potently reduced oxidative stress as evidenced by a reduction of plasma TBARS and a reduction of myocardial 3-nitrotyrosine-positive area (%). In conclusion, selective homocysteine lowering potently attenuates pressure overload-induced cardiomyopathy via reduced oxidative stress.

KEYWORDS

Heart failure, homocysteine, cardiomyopathy, transverse aortic constriction, gene transfer.

INTRODUCTION

Clinical data support the hypothesis that plasma homocysteine levels modulate myocardial biology and function. Plasma homocysteine levels are directly related to left ventricular mass and wall thickness in women but not in men in the Framingham Heart Study participants that were free of heart failure and previous myocardial infarction[1]. Elevated homocysteine levels are associated with reduced regional left ventricular systolic function determined by tagged magnetic resonance imaging in asymptomatic subjects[2]. Furthermore, left ventricular diastolic function is significantly worse in hypertensive patients with elevated levels of homocysteine compared to hypertensive patients with normal homocysteine levels[3]. Finally, plasma homocysteine levels predict heart failure incidence in patients with or without prior myocardial infarction[4-6]. Notwithstanding the consistency of the results of these clinical studies, a causal role of homocysteine in myocardial biology and function cannot be proven since residual confounding in multivariable models may occur.

The potential role of homocysteine on cardiac function is supported by *in vitro* and *ex vivo* experiments. In isolated cultured adult rat ventricular myocytes, exposure to homocysteine increases production of reactive oxygen species, impairs cardiomyocyte contractility in a concentration-dependent manner, and promotes apoptosis[7]. Cardiomyocytes isolated from hyperhomocysteinemic mice are characterized by impaired *ex vivo* contraction and relaxation[8].

Mild hyperhomocysteinemia induced in rats via chronic methionine administration or in heterozygous *cystathionine-β-synthase* (*Cbs)* deficient mice induces cardiomyocyte hypertrophy and interstitial fibrosis[9,10]. However, an analysis of *in vivo* cardiac function has not been performed in these models. Recently, we demonstrated that selective homocysteine lowering gene transfer in female C57BL/6 *low-density lipoprotein receptor* $(Ldlr)^{-/-}$ $Cbs^{+/-}$ mice fed a folate-depleted, methionine-enriched diet improves infarct healing, attenuates adverse ventricular remodelling, and significantly enhances diastolic function following permanent ligation of the left

anterior descending coronary artery[11]. However, since beneficial effects of selective homocysteine lowering gene therapy on cardiac function post-ligation of the left anterior descending coronary artery may be entirely dependent on improved infarct healing and reduced infarct expansion, direct effects of homocysteine levels on the ventricular wall cannot be proven in this model of myocardial infarction.

Transverse aortic constriction (TAC) is a commonly used model of pressure overload-induced cardiac hypertrophy and heart failure[12]. TAC initially leads to compensatory hypertrophy of the heart, but over time, the response to chronic hemodynamic overload becomes maladaptive and results in cardiac dilatation and heart failure with reduced ejection fraction. The objective of the current study is to evaluate whether plasma homocysteine has direct effects on the myocardium by investigating the impact of selective homocysteine lowering gene transfer on the development of pressure overload-induced cardiomyopathy in mice. Specifically, we investigated the hypothesis that lower homocysteine levels inhibit the development of non-ischemic cardiomyopathy via reduced oxidative stress.

MATERIALS AND METHODS

For detailed methodology, please see the Supplementary Materials and Methods in the Electronic Supplementary Material.

Construction, generation, and production of E1E3E4-deleted adenoviral gene transfer vectors- The construction of the E1E3E4-deleted adenoviral vector AdCBS, which induces hepatocyte-specific expression of cystathionine-β-synthase (CBS), has been described previously[13]. This vector contains the 1.27 kb DC172 promoter[14], consisting of an 890 bp human α_1*-antitrypsin* promoter and two copies of the 160 bp α_1*-microglobulin* enhancer, upstream of the 5' untranslated region of the human *apo A-I* gene that contains the first intron, the 1.7 kb cDNA sequence of murine *Cbs*, and 2 copies of the 774 bp *hepatic control region-1*. Hepatic overexpression of murine CBS after AdCBS gene transfer in C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice has been previously confirmed by Western blot[11]. The E1E3E4-deleted control vector Adnull does not contain an expression cassette[15]. Large scale vector production was performed as described previously[15].

In vivo experiments-All experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee of the Catholic University of Leuven (Approval number: P154/2013). Control female C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice[13] were fed standard chow (Sniff Spezialdiäten GMBH, Soest, Germany). Diet mice received a folate-depleted, methionine-enriched diet (TD00205; 0.2 mg/kg folic acid, 4.1 g/kg L-methionine; Harlan Teklad, Horst, The Netherlands) starting from the age of 12 weeks to induce hyperhomocysteinemia. Three weeks after initiation of this diet, tail vein injection was performed with 5 x 10^{10} adenoviral particles of AdCBS, with the same dose of the control vector Adnull, or with saline buffer. The equivalency of Adnull and saline injection with regard to different outcome measures has been demonstrated in several studies[16-18,11]. The experimental diet was

maintained throughout the entire duration of the experiments. Taken together, control mice were compared with diet mice and diet AdCBS mice.

To induce pressure overload, transverse aortic constriction (TAC) was performed two weeks after gene transfer or saline injection as previously described[12,19]. The sham procedure was identical except that no constriction on the aorta was applied.

Biochemical endpoint analyses-A detailed description of these quantifications is included in the Supplementary Materials and Methods.

In vivo hemodynamic measurements-Invasive hemodynamic measurements were performed 8 weeks after TAC or after sham operation as described before[17]. A detailed description can be found in the Supplementary Materials and Methods.

Histological and morphometric analysis-After hemodynamic analysis, mice were perfused via the abdominal aorta with phosphate-buffered saline (PBS) and hearts were arrested in diastole by CdCl (100 µl; 0.1 N), followed by perfusion fixation with 1% paraformaldehyde in phosphate buffered saline. After dissection, hearts were post-fixated overnight in 1% paraformaldehyde, embedded in paraffin, and 6 µm thick cross-sections at 130 µm spaced intervals were made extending from the apex to the basal part of the left ventricle. Left ventricle (LV) remodelling was assessed by morphometric analysis on mosaic images of Sirius red-stained heart cross-sections using Axiovision 4.6 software (Zeiss, Zaventem, Belgium). Whole LV area (mm^2), LV cavity area (mm^2), LV remote muscle area including the entire septum (mm^2), anterior wall thickness, and septal wall thickness were analyzed. All geometric measurements were computed in a blinded fashion from representative tissue sections of 4 separate regions and the average value was used to represent that animal for statistical purposes.

A detailed description of (immuno)histochemical analyses is included in the Supplementary Materials and Methods.

Statistical analysis-All data are expressed as means ± standard error of the means (SEM). Detailed description of statistical analysis can be retrieved in the Supplementary Materials and Methods.

RESULTS

AdCBS gene transfer selectively lowers homocysteine levels in diet mice

Plasma homocysteine levels were 15.5-fold ($p<0.001$) and 19.4-fold ($p<0.001$) higher in diet sham and diet TAC C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice compared to respective control groups (Figure 1). AdCBS gene transfer resulted in 86.4% ($p<0.001$) and 84.6% ($p<0.001$) lower homocysteine levels in diet sham mice and diet TAC mice, respectively. No significant difference of plasma homocysteine levels was observed between control groups and diet AdCBS groups (Figure 1). As illustrated in Supplementary Table 1, there was no impact of the diet, of AdCBS gene transfer, or of the TAC procedure on plasma lipoprotein cholesterol levels.

Selective homocysteine lowering gene transfer improves survival after TAC in diet mice

Comparison of Kaplan-Meier survival curves (Figure 2) showed a higher mortality rate in diet TAC mice compared to TAC control mice ($p=0.0519$) (hazard ratio for mortality 1.97, 95% CI 0.994 to 3.89). Mortality rate was significantly reduced in diet AdCBS TAC mice compared to diet TAC mice ($p<0.05$) (hazard ratio for mortality 0.495, 95% CI 0.249 to 0.985) (Figure 2). No significant difference in mortality rate was observed between control TAC mice and diet AdCBS TAC mice (Figure 2). Sham operation did not result in any mortality (data not shown).

Cardiac hypertrophy is lower and interstitial fibrosis is reduced in diet AdCBS TAC mice compared to diet TAC mice

No significant difference in heart weights (Figure 3A) or lung weights (Figure 3B) was observed between the different sham groups. The heart weight was 2.55-fold ($p<0.0001$) higher in diet TAC mice compared to diet sham mice (Figure 3A). Compared to diet TAC mice, cardiac hypertrophy was 25.3% ($p<0.01$) and 16.0% ($p<0.05$) lower in control TAC mice and diet AdCBS TAC mice, respectively. The lung weight was 2.07-fold ($p<0.001$) higher in diet TAC mice compared to diet sham mice (Figure 3B). Lung weight was 32.8% ($p<0.05$) and 15.5%

(p=NS) lower in control TAC mice and diet AdCBS TAC mice, respectively, than in diet TAC mice.

Morphometric and histological parameters of the left ventricular myocardium 8 weeks after sham operation are shown in Table 1. No significant differences were observed between the three groups with the exception of a slight increase of interstitial fibrosis in diet sham mice and diet AdCBS sham mice compared to sham control mice. Morphometric and histological parameters of the left ventricular myocardium 8 weeks after TAC are illustrated in Table 2. In agreement with the heart weight data (Figure 3A), LV wall area, septal wall thickness, and anterior wall thickness were significantly lower in control TAC mice and diet AdCBS TAC mice compared to diet TAC mice (Table 2, Supplementary Figure 1). Cardiomyocyte hypertrophy was significantly more pronounced in the diet TAC mice compared to the two other groups (Table 2, Supplementary Figure 2). Capillary density and relative vascularity were 22.9% ($p<0.05$) and 24.4% (p=NS) higher, respectively, in diet AdCBS TAC mice compared to diet TAC mice (Table 2, Supplementary Figure 2). Interstitial fibrosis was strikingly lower in control TAC mice and diet AdCBS TAC mice compared to diet TAC mice (Table 2, Supplementary Figure 2). Apoptosis in the myocardium was evaluated using immunohistochemical quantification of cleaved caspase-3. Cleaved caspase-3 positive cells were undetectable in the myocardium of control sham mice and diet AdCBS sham mice whereas cleaved caspase-3 positive cells were infrequently observed in diet sham mice (Table 1). Compared to diet TAC mice, the number of cleaved caspase-3 positive cells was reduced by 36.0% ($p<0.01$) and 32.9% ($p<0.01$) in control TAC mice and diet AdCBS TAC mice, respectively (Table 2).

Selective homocysteine lowering gene transfer improves diastolic function after TAC

Hemodynamic parameters in the left ventricle and in the aorta 8 weeks after sham operation are summarized in Table 3. There was a 15.7% ($p<0.01$) decrease of the absolute value of the peak rate of isovolumetric relaxation (dP/dt_{min}) in diet sham mice compared to control sham mice.

Following TAC, diastolic function in diet AdCBS mice was similar compared to control mice and was significantly improved compared to diet mice as evidenced by the higher absolute value of dP/dt_{min} and a lower value of the time constant of left ventricular relaxation (Table 4). End-diastolic pressure was significantly lower in control TAC mice and diet AdCBS TAC mice than in diet TAC mice (Table 4).

Hyperhomocysteinemia reduces antioxidants and decreases antioxidant defense systems in plasma

To quantify antioxidants and antioxidant defense systems, the concentration of glutathione (GSH) and cysteine and the activity of glutathione peroxidase and superoxide dismutase were determined in plasma (Supplementary Table 2 and Supplementary Table 3). The concentration of glutathione (GSH) and cysteine were significantly reduced in diet sham mice compared to control sham mice and diet sham AdCBS mice (Supplementary Table 2). Furthermore, glutathione peroxidase activity and superoxide dismutase activity were significantly lower in diet sham mice compared to the other sham groups (Supplementary Table 2). As shown in Supplementary Table 3, antioxidants and antioxidant defense systems were significantly decreased in diet TAC mice compared to control TAC mice and diet AdCBS TAC mice.

AdCBS gene transfer reduces oxidative stress

Reduction of oxidative stress may underlie the beneficial effects of selective homocysteine lowering gene transfer on cardiac hypertrophy, interstitial fibrosis, and diastolic function. Compared to diet sham mice, plasma TBARS were 32.5% ($p<0.05$) lower in both control sham mice and diet AdCBS sham mice (Figure 4A). TBARS were increased by 30.3% ($p=0.0542$), 126% ($p<0.01$), and 63.4% ($p<0.01$) in control TAC mice, diet TAC mice, and diet AdCBS TAC mice, respectively, compared to respective sham groups. Furthermore, TBARS were significantly

($p<0.001$) higher in the diet TAC mice compared to the two other TAC groups (Figure 4A). Plasma asymmetric dimethylarginine (ADMA) levels were 26.6% ($p<0.05$) and 27.4% ($p<0.05$) higher in control sham mice and in diet AdCBS sham mice, respectively, than in diet sham mice (Figure 4B). Compared to respective sham groups, a significant increase of ADMA occurred in all TAC groups but no significant difference was observed between TAC groups (Figure 4B). Representative myocardial sections immunostained for 3-nitrotyrosine staining are shown in Figure 4C. Compared to respective sham groups, 3-nitrotyrosine-positive area (%) was increased 4.65-fold ($p<0.0001$), 3.78-fold ($p<0.001$), and 2.69-fold ($p<0.0001$) in control TAC mice, diet TAC mice, and diet AdCBS TAC mice, respectively (Figure 4D). The 3-nitrotyrosine-positive area was 54.7% ($p<0.01$) and 38.6% ($p<0.05$) lower in control TAC mice and in diet AdCBS TAC mice, respectively, than in diet TAC mice. Taken together, selective homocysteine lowering gene transfer reduces myocardial oxidative stress.

DISCUSSION

The main findings of the current study are that 1) selective homocysteine lowering gene therapy in a murine model of hyperhomocysteinemia significantly reduces mortality after TAC; 2) lower plasma homocysteine levels result in reduced cardiac hypertrophy, decreased interstitial fibrosis, and improved diastolic function after TAC compared to hyperhomocysteinemic mice; 3) lower homocysteine levels result in significantly improved antioxidant defense systems both in sham mice and in TAC mice; 4) lower homocysteine plasma levels reduce oxidative stress in both sham mice and in TAC mice as evidenced by a reduction of plasma TBARS and myocardial 3-nitrotyrosine levels; 5) apoptosis in the myocardium is reduced in control TAC mice and diet AdCBS TAC mice compared to diet TAC mice.

Most studies on the effect of homocysteine on biochemical, biological, and medical end-points depend on dietary manipulations that may have effects independent of homocysteine levels. An important strength of the current study is that the decrease of homocysteine levels in mice on the folate-depleted, methionine-enriched diet was induced by selective homocysteine lowering gene transfer. Therefore, dietary effects unrelated to homocysteine lowering cannot have an impact on the end-points in our study. Most but not all parameters were very similar in control TAC mice and in diet AdCBS TAC mice indicating that in general the impact of hyperhomocysteinemia overrides any effect resulting from homocysteine-unrelated differences between standard chow and the folate-depleted, methionine-enriched diet.

Lower homocysteine levels were associated with lower lung weights at 8 weeks after TAC, which is indicative of decreased congestive heart failure. Furthermore, mortality in diet AdCBS TAC mice was significantly reduced compared to diet TAC mice, which predominantly reflects enhanced survival in the first four weeks after TAC. Early mortality after TAC may be caused by acute heart failure or by lethal arrhythmias[20].

Lower plasma homocysteine levels resulted in reduced cardiac hypertrophy after TAC. According to the classic wall stress-hypothesis of Grosmann[21], pressure overload induces myocytes to grow in width until wall thickness has increased sufficiently to normalize systolic wall stress. In this concentric hypertrophy, the fibers get thicker because sarcomeres are laid down in parallel[22]. However, hypertrophy is also influenced by load-independent factors as demonstrated by the effect of genetic background[23] and as illustrated by observations in many transgenic models[24]. Notably, these studies[23,24] highlight that there is no direct relationship between the degree of cardiac hypertrophy and cardiac function. In a mouse model with transgenic inactivation of G_q, hypertrophy from pressure loading was less than predicted and wall stress was not normalized[25]. Contractility in these transgenic mice was better than in wild-type mice with more hypertrophy and normalization of wall stress. On the other hand, dual specificity mitogen-activated protein kinase kinase 1 (MEK1) transgenic mice demonstrated concentric hypertrophy without signs of cardiomyopathy or lethality up to 12 months of age[26]. These MEK1 transgenic mice showed a dramatic increase in cardiac function as quantified by echocardiography and as measured in isolated working heart preparations[26]. Here, we demonstrate that reduced cardiac hypertrophy following lowering of plasma homocysteine levels is associated with improved diastolic function and a trend for an improvement of systolic function. The relation between cardiac hypertrophy and cardiac function can also be analyzed from a clinical perspective. Left ventricular hypertrophy has an important prognostic value in patients. Hypertensive patients with concentric left ventricular hypertrophy have the highest incidence of cardiovascular events including death[27]. In patients with severe aortic stenosis, the degree of left ventricular hypertrophy independently predicts heart failure regardless of the degree of flow restriction imposed by the valve pathology[28]. Taken together, less pronounced left ventricular hypertrophy as observed following homocysteine lowering may be beneficial and may contribute to the improved cardiac function. In this regard, homocysteine levels in patients correlate with

brain natriuretic peptide[29] and the N-terminal of the prohormone brain natriuretic peptide[30], biomarkers of chronic heart failure.

Pressure overload resulted in oxidative stress in all three TAC groups as evidenced by elevated plasma TBARS and 3-nitrotyrosine immunostaining. Oxidative stress refers to situations characterized by an imbalance between the production of reactive oxygen species and antioxidant defenses leading to oxidative modification of numerous biological substrates[31]. A chronic increase in reactive oxygen species plays a critical role in the development and progression of heart failure. Markers of oxidative stress are increased in patients with heart failure and correlate with the severity of cardiac dysfunction[32]. Reactive oxygen species can induce DNA damage, protein damage, and disrupt cell membranes. They may contribute to cardiomyocyte hypertrophy and lead to cardiomyocyte apoptosis. Reactive oxygen species can be produced through electron leakage from the mitochondria during oxidative phosphorylation. Alternatively, they are generated by several enzymes including nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, xanthine oxidase, and nitric oxide synthase[33].

Hyperhomocysteinemia augmented oxidative stress in TAC mice. Antioxidants (glutathione, cysteine) and antioxidant defense systems (glutathione peroxidase, superoxide dismutase) were significantly lower in hyperhomocysteinemic mice. CBS converts homocysteine to cystathionine. The increase of cysteine and glutathione following AdCBS gene transfer reflects increased substrate availability of metabolic pathways downstream of cystathionine. Handy *et al*.[34] previously demonstrated that homocysteine decreases glutathione peroxidase activity by altering the specific translational mechanism essential for the synthesis of this selenocysteine-containing protein. Superoxide dismutase protein levels in the heart were reduced in a dog model[35] and in a rat model of chronic hyperhomocysteinemia[36]. Production of reactive oxygen species is increased by hyperhomocysteinemia. Auto-oxidation of homocysteine generates reactive oxygen species including superoxide, hydrogen peroxide, and hydroxyl radicals, and also directly

contributes to the depletion of nitric oxide via formation of peroxynitrite ($ONOO^-$)[37,38]. In addition, hyperhomocysteinemia has been shown to induce NADPH-oxidase 2 (Nox2) [35] and Nox 4 in cardiomyocytes[39]. Furthermore, hyperhomocysteinemia promotes expression of inducible NO synthase (iNOS), further increasing superoxide radical formation[40]. In isolated cultured adult rat ventricular myocytes, exposure to homocysteine for 24 hours impaired cardiomyocyte contractility in a concentration-dependent manner and promoted apoptosis[7]. These effects were associated with activation of p38 mitogen-activated protein kinase and increased production of reactive oxygen species. Inhibition of p38 mitogen-activated protein kinase prevented all of the effects of homocysteine[7]. Furthermore, oxidative stress activates Ca^{2+}-calmodulin kinase II. Under physiological conditions, this enzyme couples increases in intracellular Ca^{2+} to activation of ion channels by phosphorylation of a large and diverse set of effector targets[41]. As a result of oxidative stress, $Ca2^{+}$-calmodulin kinase II activity is sustained, independent of Ca^{2+} and calmodulin amounts, which may induce cardiac arrhythmias[42]. This may contribute to increased mortality in diet TAC mice. This enzyme also provides a link between increased oxidative stress and increased apoptosis in diet TAC mice since knockdown of Ca^{2+}-calmodulin kinase II in cardiac myocytes reduces apoptosis[42]. Oxidative stress may also contribute to increased interstitial fibrosis in diet TAC mice. Exposure of cardiac fibroblasts to superoxide anion enhances the production of transforming growth factor-β1 (TGF-β1)[43,44], which is a potent fibrogenic cytokine. Recently, up-regulation of Nox4 in the myocardium has been directly linked to increased interstitial fibrosis[45].

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide (NO) synthase (NOS). ADMA is produced from the proteolysis of proteins that contain dimethylated arginine residues. ADMA levels were quite surprisingly lower in diet sham mice compared to the two sham groups with lower homocysteine levels. This observation may be the result of a large decrease in the hepatic methylation capacity (low *S*-adenosylmethionine-to-*S*-

adenosylhomocysteine ratio) induced by the high-methionine low-folate diet in *Cbs*$^{+/-}$ mice [46]. ADMA levels were increased in all three TAC groups compared to respective sham groups. These increments reflect the increased oxidative stress induced by TAC. Indeed, increased oxidative stress results in downregulation of dimethylarginine dimethylaminohydrolase[47-49]. Since dimethylarginine dimethylaminohydrolase hydrolyzes ADMA to citrulline and dimethylamine, reduced activity of this enzyme results in increased ADMA levels. However, in light of similar ADMA levels in the three TAC groups, our data do not indicate that ADMA levels contribute significantly to the more pronounced deterioration of cardiac function in diet TAC mice compared to the two TAC groups with low homocysteine levels.

In conclusion, selective homocysteine lowering gene therapy in a murine model of hyperhomocysteinemia significantly reduces mortality, attenuates cardiac hypertrophy, decreases interstitial fibrosis, reduces apoptosis, and enhances diastolic function after TAC. These beneficial effects are likely closely linked to a profound reduction of oxidative stress secondary to the decrease of homocysteine levels.

ACKNOWLEDGEMENTS

This work was supported by Onderzoekstoelagen grant OT/13/090 of the KU Leuven and by grant G0A3114N of the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen.

DISCLOSURES

None of the authors has a conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Institutional Animal Care and Research Advisory Committee of the Catholic University of Leuven. Approval number: P154/2013).

REFERENCES

1. Sundstrom J, Sullivan L, Selhub J, Benjamin EJ, D'Agostino RB, Jacques PF, Rosenberg IH, Levy D, Wilson PW, Vasan RS (2004) Relations of plasma homocysteine to left ventricular structure and function: the Framingham Heart Study. Eur Heart J 25 (6):523-530. doi:10.1016/j.ehj.2004.01.008

2. Nasir K, Tsai M, Rosen BD, Fernandes V, Bluemke DA, Folsom AR, Lima JA (2007) Elevated homocysteine is associated with reduced regional left ventricular function: the Multi-Ethnic Study of Atherosclerosis. Circulation 115 (2):180-187. doi:10.1161/CIRCULATIONAHA.106.633750

3. Ruhui L, Jinfa J, Jiahong X, Wenlin M (2014) Influence of hyperhomocysteinemia on left ventricular diastolic function in Chinese patients with hypertension. Herz. doi:10.1007/s00059-014-4098-x

4. Washio T, Nomoto K, Watanabe I, Tani S, Nagao K, Hirayama A (2011) Relationship between plasma homocysteine levels and congestive heart failure in patients with acute myocardial infarction. Homocysteine and congestive heart failure. Int Heart J 52 (4):224-228

5. Vasan RS, Beiser A, D'Agostino RB, Levy D, Selhub J, Jacques PF, Rosenberg IH, Wilson PW (2003) Plasma homocysteine and risk for congestive heart failure in adults without prior myocardial infarction. Jama 289 (10):1251-1257

6. May HT, Alharethi R, Anderson JL, Muhlestein JB, Reyna SP, Bair TL, Horne BD, Kfoury AG, Carlquist JF, Renlund DG (2007) Homocysteine levels are associated with increased risk of congestive heart failure in patients with and without coronary artery disease. Cardiology 107 (3):178-184. doi:10.1159/000095344

7. Wang X, Cui L, Joseph J, Jiang B, Pimental D, Handy DE, Liao R, Loscalzo J (2012) Homocysteine induces cardiomyocyte dysfunction and apoptosis through p38 MAPK-mediated increase in oxidant stress. J Mol Cell Cardiol 52 (3):753-760. doi:10.1016/j.yjmcc.2011.12.009

8. Moshal KS, Tipparaju SM, Vacek TP, Kumar M, Singh M, Frank IE, Patibandla PK, Tyagi N, Rai J, Metreveli N, Rodriguez WE, Tseng MT, Tyagi SC (2008) Mitochondrial matrix metalloproteinase activation decreases myocyte contractility in hyperhomocysteinemia. Am J Physiol Heart Circ Physiol 295 (2):H890-897. doi:10.1152/ajpheart.00099.2008

9. Raaf L, Noll C, Cherifi Mel H, Samuel JL, Delcayre C, Delabar JM, Benazzoug Y, Janel N (2011) Myocardial fibrosis and TGFB expression in hyperhomocysteinemic rats. Mol Cell Biochem 347 (1-2):63-70. doi:10.1007/s11010-010-0612-5

10. Kundu S, Kumar M, Sen U, Mishra PK, Tyagi N, Metreveli N, Lominadze D, Rodriguez W, Tyagi SC (2009) Nitrotyrosinylation, remodeling and endothelial-myocyte uncoupling in iNOS, cystathionine beta synthase (CBS) knockouts and iNOS/CBS double knockout mice. J Cell Biochem 106 (1):119-126. doi:10.1002/jcb.21982

11. Muthuramu I, Jacobs F, Singh N, Gordts SC, De Geest B (2013) Selective homocysteine lowering gene transfer improves infarct healing, attenuates remodelling, and enhances diastolic function after myocardial infarction in mice. PLoS One 8 (5):e63710. doi:10.1371/journal.pone.0063710

12. Buys ES, Raher MJ, Blake SL, Neilan TG, Graveline AR, Passeri JJ, Llano M, Perez-Sanz TM, Ichinose F, Janssens S, Zapol WM, Picard MH, Bloch KD, Scherrer-Crosbie M (2007) Cardiomyocyte-restricted restoration of nitric oxide synthase 3 attenuates left ventricular remodeling after chronic pressure overload. Am J Physiol Heart Circ Physiol 293 (1):H620-627

13. Jacobs F, Van Craeyveld E, Muthuramu I, Gordts SC, Emmerechts J, Hoylaerts M, Herijgers P, De Geest B (2011) Correction of endothelial dysfunction after selective homocysteine lowering gene therapy reduces arterial thrombogenicity but has no effect on atherogenesis. J Mol Med 89 (10):1051-1058. doi:10.1007/s00109-011-0778-7

14. Jacobs F, Snoeys J, Feng Y, Van Craeyveld E, Lievens J, Armentano D, Cheng SH, De Geest B (2008) Direct comparison of hepatocyte-specific expression cassettes following adenoviral and nonviral hydrodynamic gene transfer. Gene Ther 15 (8):594-603
15. Van Linthout S, Lusky M, Collen D, De Geest B (2002) Persistent hepatic expression of human apo A-I after transfer with a helper-virus independent adenoviral vector. Gene Ther 9 (22):1520-1528.
16. Gordts SC, Van Craeyveld E, Muthuramu I, Singh N, Jacobs F, De Geest B (2012) Lipid Lowering and HDL Raising Gene Transfer Increase Endothelial Progenitor Cells, Enhance Myocardial Vascularity, and Improve Diastolic Function. PLoS One 7 (10):e46849. doi:10.1371/journal.pone.0046849
17. Van Craeyveld E, Jacobs F, Gordts SC, De Geest B (2012) Low-density lipoprotein receptor gene transfer in hypercholesterolemic mice improves cardiac function after myocardial infarction. Gene Ther 19 (8):860-871. doi:10.1038/gt.2011.147
18. Van Craeyveld E, Gordts SC, Nefyodova E, Jacobs F, De Geest B (2011) Regression and stabilization of advanced murine atherosclerotic lesions: a comparison of LDL lowering and HDL raising gene transfer strategies. J Mol Med 89 (6):555-567. doi:10.1007/s00109-011-0722-x
19. Hu P, Zhang D, Swenson L, Chakrabarti G, Abel ED, Litwin SE (2003) Minimally invasive aortic banding in mice: effects of altered cardiomyocyte insulin signaling during pressure overload. Am J Physiol Heart Circ Physiol 285 (3):H1261-1269. doi:10.1152/ajpheart.00108.2003
20. Yasuno S, Kuwahara K, Kinoshita H, Yamada C, Nakagawa Y, Usami S, Kuwabara Y, Ueshima K, Harada M, Nishikimi T, Nakao K (2013) Angiotensin II type 1a receptor signalling directly contributes to the increased arrhythmogenicity in cardiac hypertrophy. Br J Pharmacol 170 (7):1384-1395. doi:10.1111/bph.12328
21. Grossman W, Jones D, McLaurin LP (1975) Wall stress and patterns of hypertrophy in the human left ventricle. J Clin Invest 56 (1):56-64. doi:10.1172/JCI108079
22. Cohn JN (1993) Post-MI remodeling. Clin Cardiol 16 (5 Suppl 2):II21-24
23. Barrick CJ, Dong A, Waikel R, Corn D, Yang F, Threadgill DW, Smyth SS (2009) Parent-of-origin effects on cardiac response to pressure overload in mice. Am J Physiol Heart Circ Physiol 297 (3):H1003-1009. doi:10.1152/ajpheart.00896.2008
24. Heineke J, Molkentin JD (2006) Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7 (8):589-600. doi:10.1038/nrm1983
25. Esposito G, Rapacciuolo A, Naga Prasad SV, Takaoka H, Thomas SA, Koch WJ, Rockman HA (2002) Genetic alterations that inhibit in vivo pressure-overload hypertrophy prevent cardiac dysfunction despite increased wall stress. Circulation 105 (1):85-92
26. Bueno OF, De Windt LJ, Tymitz KM, Witt SA, Kimball TR, Klevitsky R, Hewett TE, Jones SP, Lefer DJ, Peng CF, Kitsis RN, Molkentin JD (2000) The MEK1-ERK1/2 signaling pathway promotes compensated cardiac hypertrophy in transgenic mice. Embo J 19 (23):6341-6350. doi:10.1093/emboj/19.23.6341
27. Koren MJ, Devereux RB, Casale PN, Savage DD, Laragh JH (1991) Relation of left ventricular mass and geometry to morbidity and mortality in uncomplicated essential hypertension. Ann Intern Med 114 (5):345-352
28. Kupari M, Turto H, Lommi J (2005) Left ventricular hypertrophy in aortic valve stenosis: preventive or promotive of systolic dysfunction and heart failure? Eur Heart J 26 (17):1790-1796. doi:10.1093/eurheartj/ehi290
29. Herrmann W, Herrmann M, Joseph J, Tyagi SC (2007) Homocysteine, brain natriuretic peptide and chronic heart failure: a critical review. Clin Chem Lab Med 45 (12):1633-1644. doi:10.1515/CCLM.2007.360
30. Gueant Rodriguez RM, Spada R, Pooya S, Jeannesson E, Moreno Garcia MA, Anello G, Bosco P, Elia M, Romano A, Alberto JM, Juilliere Y, Gueant JL (2013) Homocysteine predicts

increased NT-pro-BNP through impaired fatty acid oxidation. Int J Cardiol 167 (3):768-775. doi:10.1016/j.ijcard.2012.03.047
31. Sirker A, Zhang M, Murdoch C, Shah AM (2007) Involvement of NADPH oxidases in cardiac remodelling and heart failure. Am J Nephrol 27 (6):649-660. doi:10.1159/000109148
32. McMurray J, Chopra M, Abdullah I, Smith WE, Dargie HJ (1993) Evidence of oxidative stress in chronic heart failure in humans. Eur Heart J 14 (11):1493-1498
33. Sorescu D, Griendling KK (2002) Reactive oxygen species, mitochondria, and NAD(P)H oxidases in the development and progression of heart failure. Congest Heart Fail 8 (3):132-140
34. Handy DE, Zhang Y, Loscalzo J (2005) Homocysteine down-regulates cellular glutathione peroxidase (GPx1) by decreasing translation. J Biol Chem 280 (16):15518-15525. doi:10.1074/jbc.M501452200
35. Suematsu N, Ojaimi C, Kinugawa S, Wang Z, Xu X, Koller A, Recchia FA, Hintze TH (2007) Hyperhomocysteinemia Alters Cardiac Substrate Metabolism by Impairing Nitric Oxide Bioavailability Through Oxidative Stress. Circulation
36. Kolling J, Scherer EB, da Cunha AA, da Cunha MJ, Wyse AT (2011) Homocysteine induces oxidative-nitrative stress in heart of rats: prevention by folic acid. Cardiovasc Toxicol 11 (1):67-73. doi:10.1007/s12012-010-9094-7
37. Dayal S, Arning E, Bottiglieri T, Boger RH, Sigmund CD, Faraci FM, Lentz SR (2004) Cerebral vascular dysfunction mediated by superoxide in hyperhomocysteinemic mice. Stroke; a journal of cerebral circulation 35 (8):1957-1962
38. Eberhardt RT, Forgione MA, Cap A, Leopold JA, Rudd MA, Trolliet M, Heydrick S, Stark R, Klings ES, Moldovan NI, Yaghoubi M, Goldschmidt-Clermont PJ, Farber HW, Cohen R, Loscalzo J (2000) Endothelial dysfunction in a murine model of mild hyperhomocyst(e)inemia. J Clin Invest 106 (4):483-491
39. Mishra PK, Tyagi N, Kundu S, Tyagi SC (2009) MicroRNAs are involved in homocysteine-induced cardiac remodeling. Cell Biochem Biophys 55 (3):153-162. doi:10.1007/s12013-009-9063-6
40. Ungvari Z, Csiszar A, Edwards JG, Kaminski PM, Wolin MS, Kaley G, Koller A (2003) Increased superoxide production in coronary arteries in hyperhomocysteinemia: role of tumor necrosis factor-alpha, NAD(P)H oxidase, and inducible nitric oxide synthase. Arteriosclerosis, thrombosis, and vascular biology 23 (3):418-424
41. Xie LH, Chen F, Karagueuzian HS, Weiss JN (2009) Oxidative-stress-induced afterdepolarizations and calmodulin kinase II signaling. Circ Res 104 (1):79-86. doi:10.1161/CIRCRESAHA.108.183475
42. Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJ, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ, Anderson ME (2008) A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. Cell 133 (3):462-474. doi:10.1016/j.cell.2008.02.048
43. Cucoranu I, Clempus R, Dikalova A, Phelan PJ, Ariyan S, Dikalov S, Sorescu D (2005) NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. Circ Res 97 (9):900-907. doi:10.1161/01.RES.0000187457.24338.3D
44. Zhao W, Zhao T, Chen Y, Ahokas RA, Sun Y (2008) Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats. Mol Cell Biochem 317 (1-2):43-50. doi:10.1007/s11010-008-9803-8
45. Zhao QD, Viswanadhapalli S, Williams P, Shi Q, Tan C, Yi X, Bhandari B, Abboud HE (2015) NADPH Oxidase 4 Induces Cardiac Fibrosis and Hypertrophy Through Activating Akt/mTOR and NFkappaB Signaling Pathways. Circulation 131 (7):643-655. doi:10.1161/CIRCULATIONAHA.114.011079

46. Dayal S, Rodionov RN, Arning E, Bottiglieri T, Kimoto M, Murry DJ, Cooke JP, Faraci FM, Lentz SR (2008) Tissue-specific downregulation of dimethylarginine dimethylaminohydrolase in hyperhomocysteinemia. Am J Physiol Heart Circ Physiol 295 (2):H816-825. doi:10.1152/ajpheart.01348.2007
47. Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP (1999) Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. Circulation 99 (24):3092-3095
48. Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP (2002) Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. Circulation 106 (8):987-992
49. Stuhlinger MC, Tsao PS, Her JH, Kimoto M, Balint RF, Cooke JP (2001) Homocysteine impairs the nitric oxide synthase pathway: role of asymmetric dimethylarginine. Circulation 104 (21):2569-2575

LEGENDS TO THE FIGURES

Figure 1. **Plasma homocysteine levels in control, diet, and diet AdCBS mice.** Sham mice and TAC mice are indicated by open bars and closed bars, respectively. Plasma samples were obtained at day 56 after sham operation or TAC.

Figure 2. **Kaplan-Meier survival curves after TAC**. Control mice, diet mice, and diet AdCBS mice are indicated by black lines, red lines, and blue lines respectively. Survival curves were compared by log-rank test.

Figure 3. Heart weights (panel A) and lung weights (panel B) in control, diet, and diet AdCBS mice. Sham mice and TAC mice are indicated by open bars and closed bars, respectively. Data reflect wet weights at day 56 after sham operation or TAC.

Figure 4. Quantification of oxidative stress in sham mice and in TAC mice. Sham mice and TAC mice are indicated by open bars and closed bars, respectively. (A) Plasma TBARS expressed as plasma malondialdehyde equivalents. (B) Plasma asymmetric dimethylarginine levels. (C) Representative photomicrographs showing myocardial sections stained for 3-nitrotyrosine. Scale bar represents 100 μm. (D) Percentage of 3-nitrotyrosine-positive area in the myocardium.

Figure 1
Click here to download high resolution image

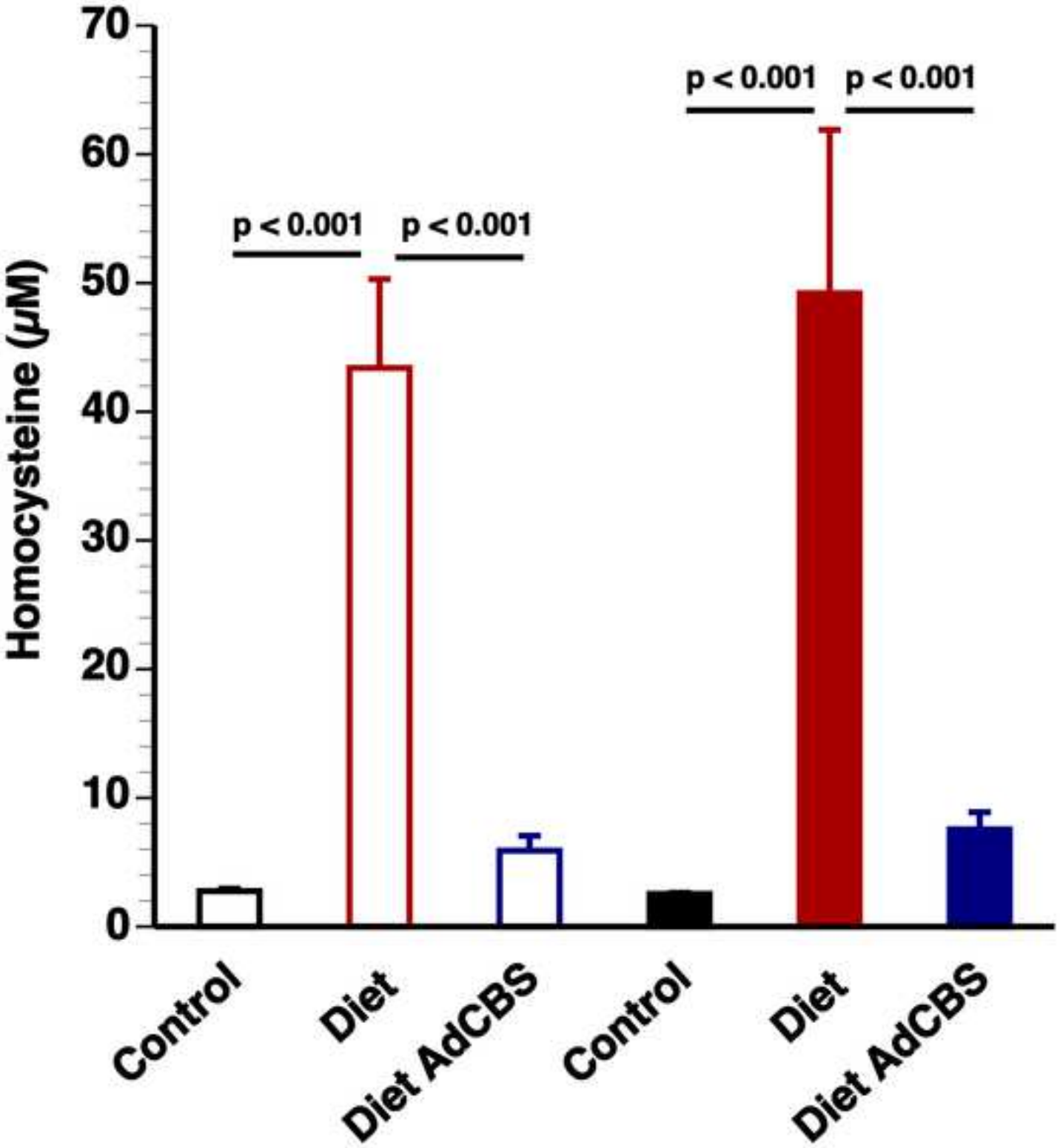


Figure 2
Click here to download high resolution image

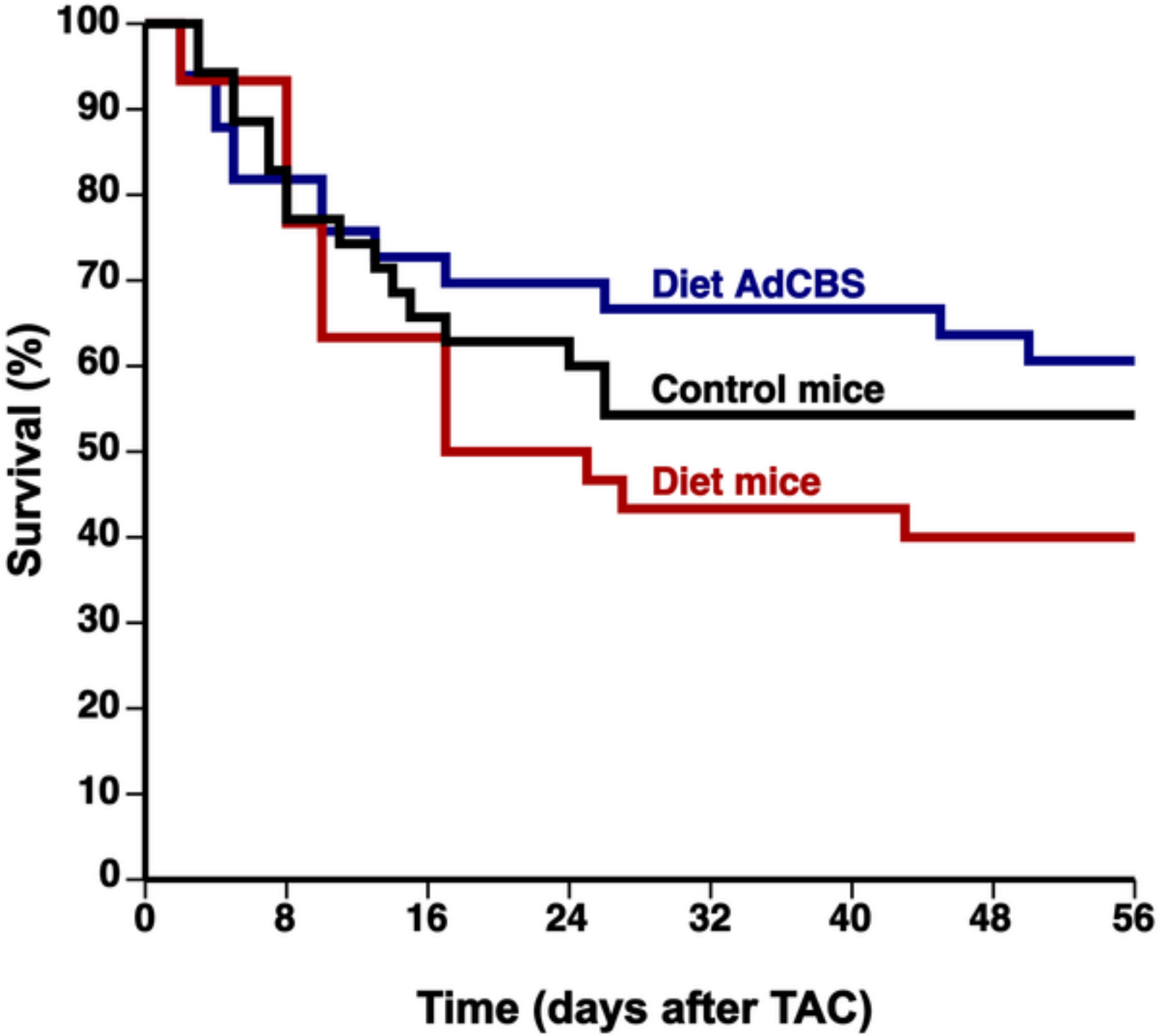


Figure 3
Click here to download high resolution image

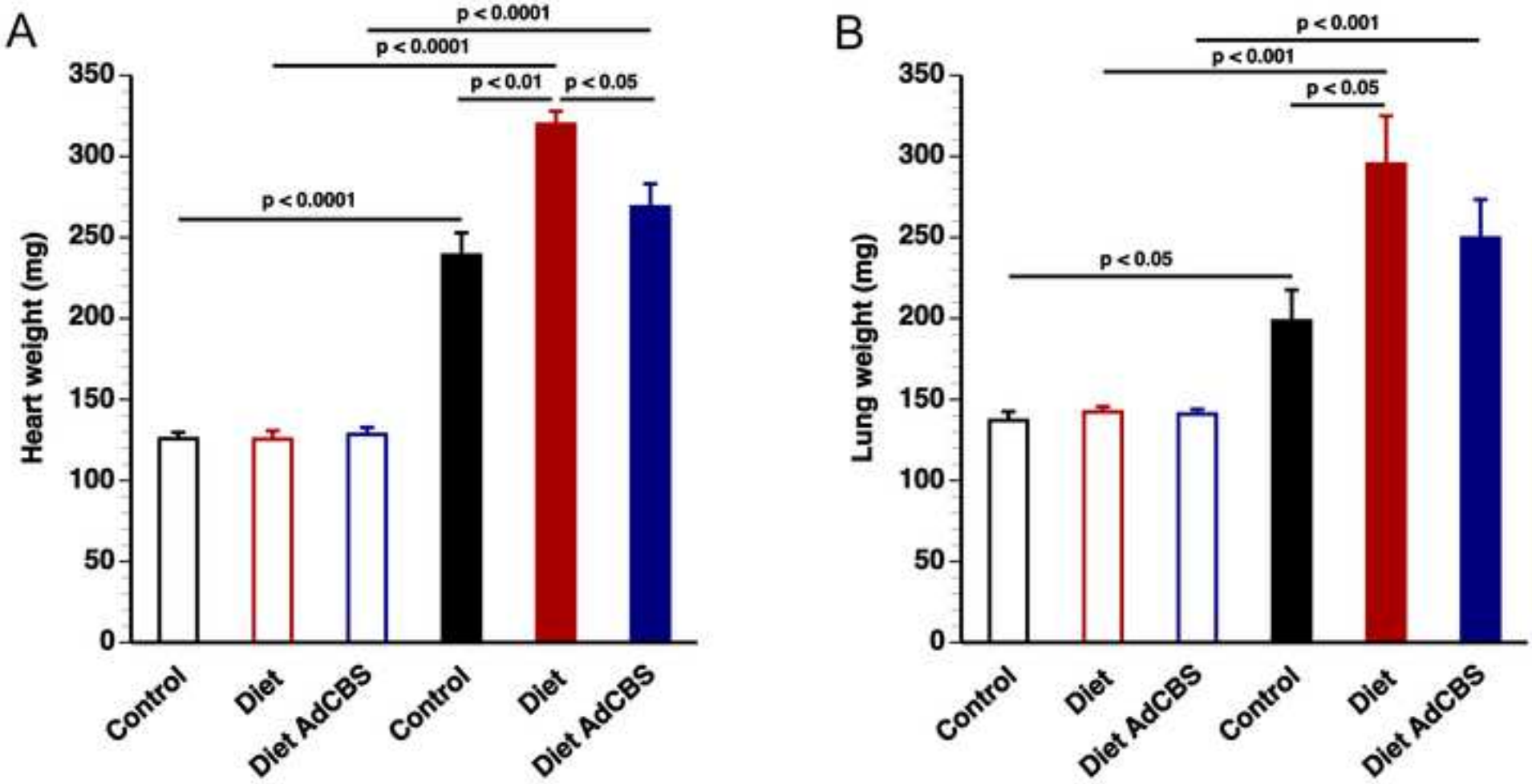
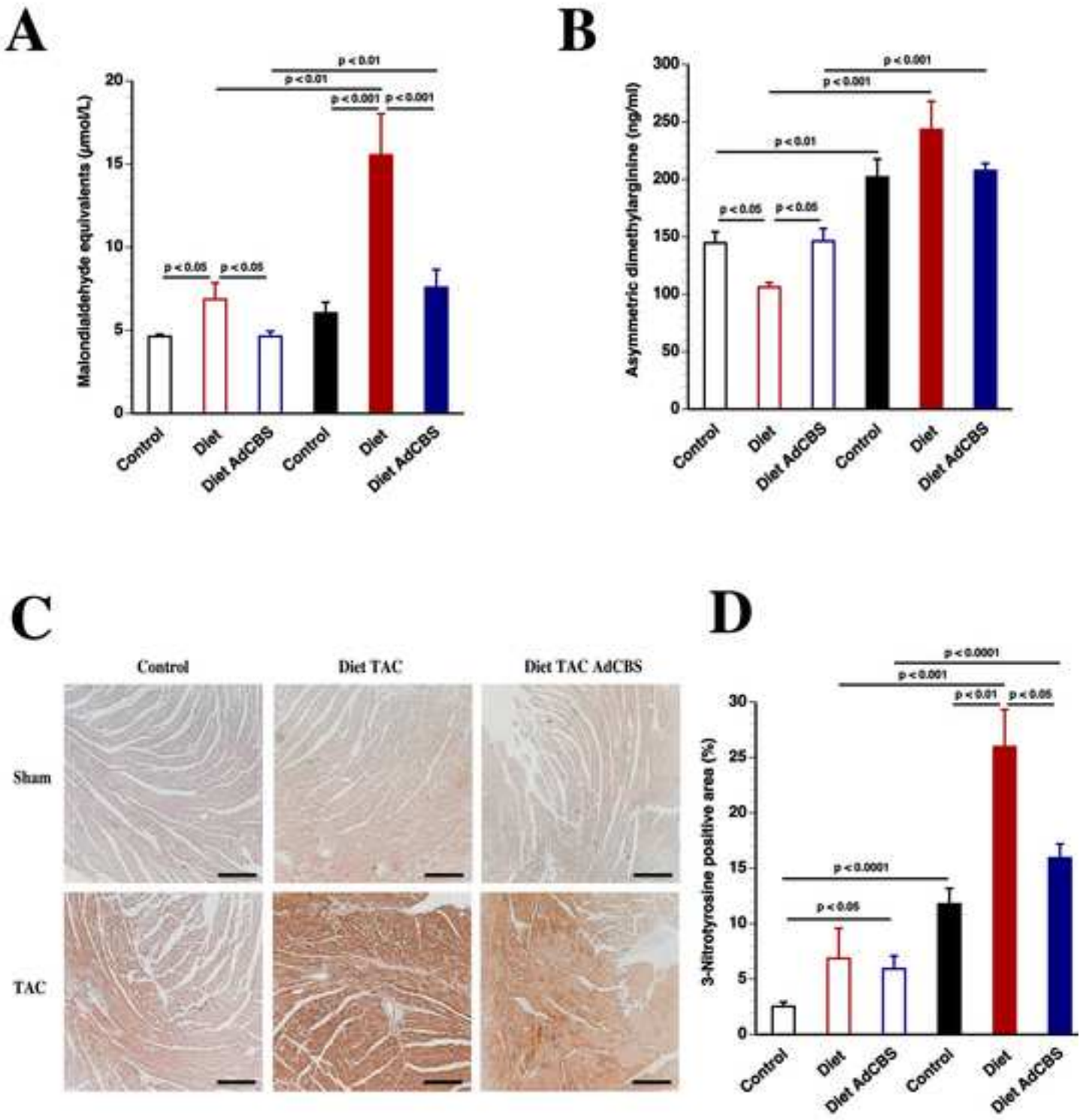
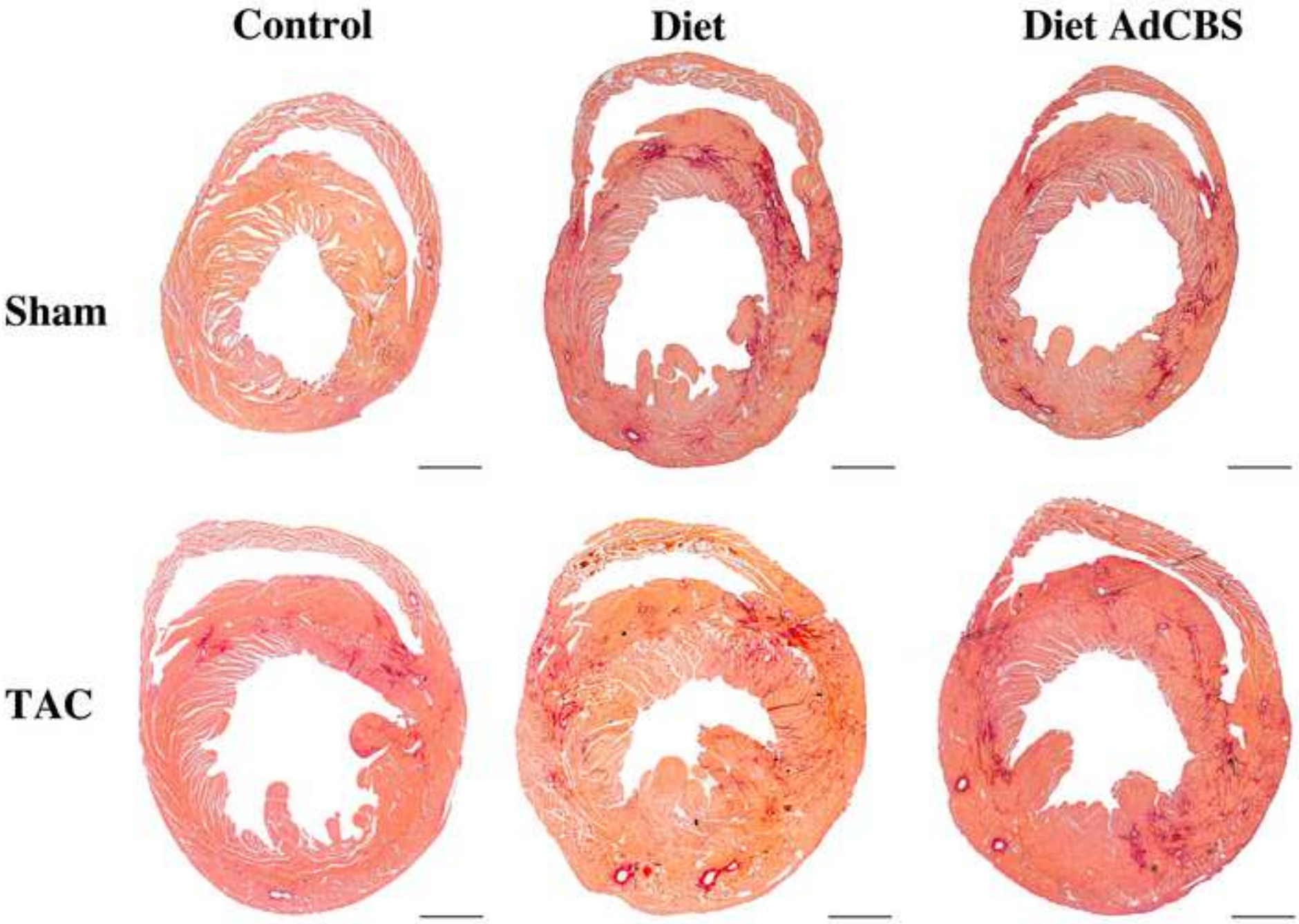


Figure 4
Click here to download high resolution image



Supplemental Figure 1
Click here to download high resolution image



Supplemental Figure 2
Click here to download high resolution image

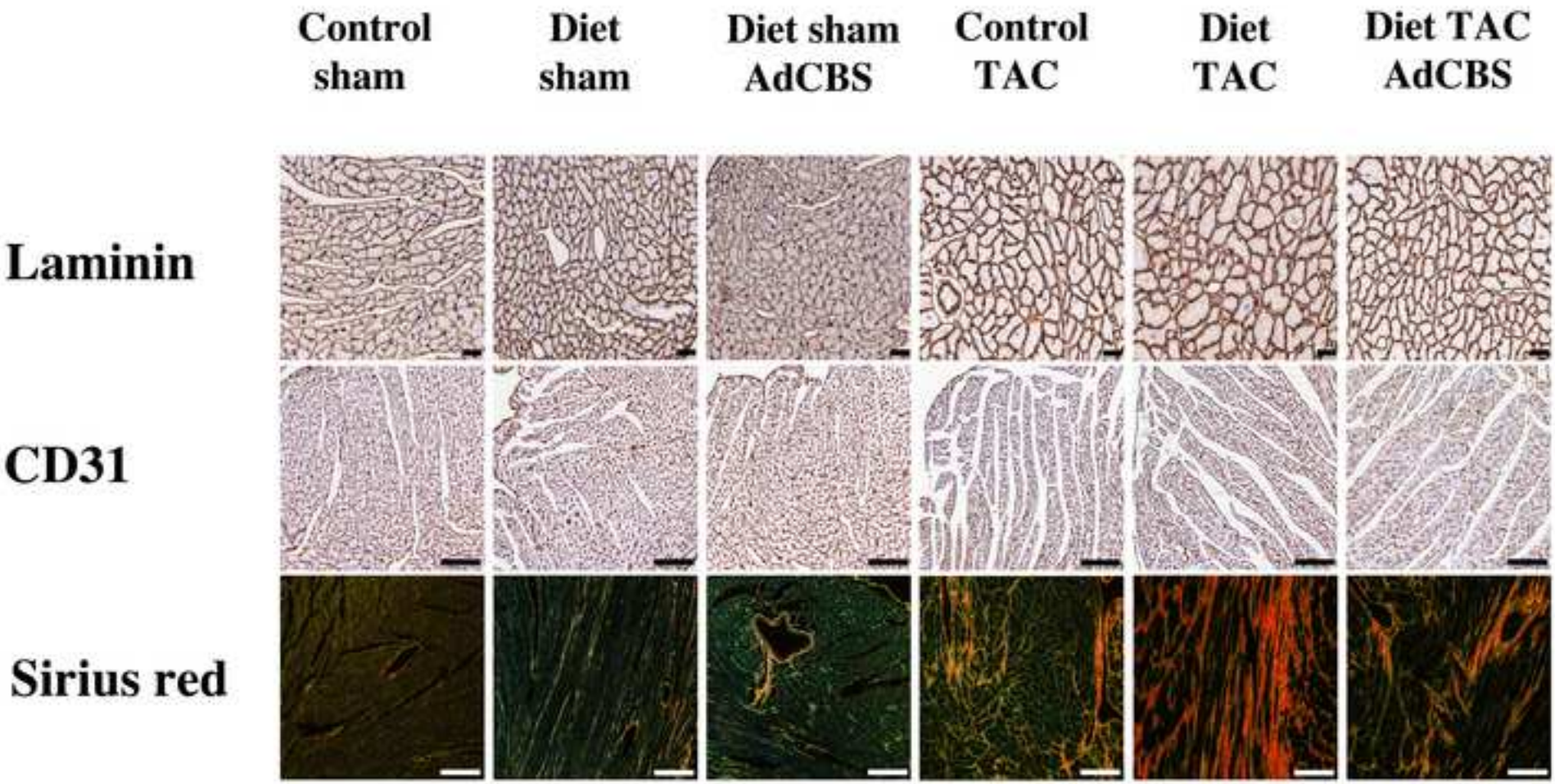


Table 1

Table 1. Morphometric and histological parameters of the left ventricular myocardium 8 weeks after sham operation in female C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice.

	Control sham	Diet sham	Diet sham AdCBS
Number of mice	12	17	24
LV wall area (mm^2)	8.89 ± 0.30	9.07 ± 0.30	8.95 ± 0.11
Septal wall thickness (µm)	1100 ± 30	1110 ± 30	1090 ± 20
Anterior wall thickness (µm)	1140 ± 30	1130 ± 30	1120 ± 10
Cardiomyocyte cross-sectional area (μm^2)	215 ± 12	184 ± 8	186 ± 6
Cardiomyocyte density (number/mm^2)	5110 ± 200	5000 ± 220	5320 ± 160
Capillary density (number/mm^2)	6760 ± 250	6320 ± 170	6610 ± 150
Relative vascularity (μm^{-2})	0.00634 ± 0.00031	0.00705 ± 0.00023	0.00684 ± 0.00020
Interstitial fibrosis (%)	2.69 ± 0.20	4.01 ± 1.3°°	4.07 ± 0.44°
Perivascular fibrosis (ratio)	0.469 ± 0.017	0.502 ± 0.026	0.489 ± 0.020
Cleaved caspase 3 positive cells (number/mm^2)	0.00 ± 0.01	2.09 ± 1.33°	0.00 ± 0.01*

Control mice were fed standard chow. The Diet (TD00205) is a folate-depleted, methionine-enriched diet.
Data are expressed as means ± SEM. °: $p<0.05$; °°:$p<0.01$ versus Control sham. *:$p<0.05$ versus diet sham.

Table 2

Table 2. Morphometric and histological parameters of the left ventricular myocardium 8 weeks after TAC in female C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice.

	Control TAC	Diet TAC	Diet TAC AdCBS
Number of mice	17	13	23
LV wall area (mm^2)	11.1 ± 0.4	13.8 ± 0.3°°°	12.3 ± 0.4*
Septal wall thickness (µm)	1280 ± 30	1460 ± 30°°°	1340 ± 30**
Anterior wall thickness (µm)	1340 ± 30	1510 ± 40°°	1320 ± 30***
Cardiomyocyte cross-sectional area ($µm^2$)	417 ± 25	503 ± 18°	420 ± 25*
Cardiomyocyte density (number/mm^2)	3140 ± 140	2500 ± 100°	3210 ± 210*
Capillary density (number/mm^2)	4560 ± 190	4360 ± 230	5360 ± 280*
Relative vascularity ($µm^{-2}$)	0.00372 ± 0.00024	0.00353 ± 0.00019	0.00439 ± 0.00027
Interstitial fibrosis (%)	11.3 ± 0.6	17.1 ± 1.3°	9.21 ± 0.89***
Perivascular fibrosis (ratio)	0.567 ± 0.040	0.727 ± 0.042°	0.529 ± 0.020**
Cleaved caspase 3 positive cells (number/mm^2)	15.8 ± 1.5	24.7 ± 2.0°°	16.6 ± 1.0**

Control mice were fed standard chow. The diet (TD00205) is a folate-depleted, methionine-enriched diet.
Data are expressed as means ± SEM. °: p<0.05; °°:p<0.01; °°°:p<0.001 versus Control TAC. *:p<0.05; **:p<0.01; ***:p<0.001 versus Diet TAC.

Table 3

Table 3. Hemodynamic parameters in the left ventricle and in the aorta 8 weeks after sham operation in female C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice.

	Control sham	Diet sham	Diet sham AdCBS
Number of mice	8	17	20
LEFT VENTRICLE			
Peak systolic pressure (mm Hg)	103 ± 3	99.1 ± 1.4	99.4 ± 2.3
End-diastolic pressure (mm Hg)	3.07 ± 0.45	1.69 ± 0.49	1.69 ± 0.36
dP/dt max (mm Hg/ms)	13.6 ± 0.8	11.6 ± 0.6	11.9 ± 0.4
dP/dt min (mm Hg/ms)	-11.4 ± 0.5	-9.61 ± 0.36°°	-10.1 ± 0.2
Tau (ms)	4.46 ± 0.19	4.83 ± 0.19	4.83 ± 0.12
Heart rate (bpm)	598 ± 18	611 ± 10	571 ± 11
AORTA			
Mean pressure (mm Hg)	97.7 ± 2.6	101 ± 5	98.7 ± 3.3
Peak systolic pressure (mm Hg)	101 ± 2	98.7 ± 1.7	99.3 ± 2.9
Peak diastolic pressure (mm Hg)	64.9 ± 3.4	69.6 ± 2.9	67.6 ± 4.0

Control mice were fed standard chow. The diet (TD00205) is a folate-depleted, methionine-enriched diet. Hemodynamic analysis was performed at the age of 25 weeks. Data are expressed as means ± SEM. °°:$p<0.01$ versus Control sham.

Table 4

Table 4. Hemodynamic parameters in the left ventricle and in the aorta 8 weeks after TAC in female C57BL/6 *Ldlr*$^{-/-}$ *Cbs*$^{+/-}$ mice.

	Control TAC	Diet TAC	Diet TAC AdCBS
Number of mice	14	10	12
LEFT VENTRICLE			
Peak systolic pressure (mm Hg)	162 ± 6	159 ± 8	164 ± 4
End-diastolic pressure (mm Hg)	1.88 ± 0.55	6.26 ± 0.80°°°	3.77 ± 0.37**
dP/dt_{max} (mm Hg/ms)	9.48 ± 0.43	8.55 ± 0.71	9.55 ± 0.36
dP/dt_{min} (mm Hg/ms)	-9.25 ± 0.58	-7.78 ± 0.41°	-9.02 ± 0.25*
Tau (ms)	5.72 ± 0.22	7.07 ± 0.30°°	5.91 ± 0.24*
Heart rate (bpm)	576 ± 21	580 ± 31	585 ± 15
AORTA			
Mean pressure (mm Hg)	97.7 ± 2.6	101 ± 5	98.2 ± 3.8
Peak systolic pressure (mm Hg)	159 ± 5	157 ± 8	163 ± 5
Peak diastolic pressure (mm Hg)	59.5 ± 3.5	67.2 ± 4.7	58.6 ± 2.9

Control mice were fed standard chow. The diet (TD00205) is a folate-depleted, methionine-enriched diet. Hemodynamic analysis was performed at the age of 25 weeks. Data are expressed as means ± SEM. °:p<0.05; °°:p<0.01; °°°:p<0.001 versus Control TAC. *:p<0.05; **:p<0.01 versus Diet TAC.

Supplementary Material

Click here to download Supplementary Material: Supplemental materials final.doc